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—Original—

Permeability of Mouse Oocytes and Embryos at Various Developmental Stages to Five Cryoprotectants

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Abstract. To assess the permeability of mouse oocytes and embryos, matured oocytes and embryos at various stages of development were placed in five cryoprotectant solutions at 25 C for 25 min. From the cross-sectional areas of the oocytes/embryos, the relative change in volume was analyzed. In oocytes, shrinkage was least extensive and recovery was quickest in the propylene glycol solution, showing that propylene glycol permeates the oocytes most rapidly. Dimethyl sulfoxide, acetamide, and ethylene glycol permeated the oocytes slightly more slowly than propylene glycol. The oocytes in glycerol shrunk extensively and then expanded marginally, indicating slow permeation. The volume changes of 1-cell and 2-cell embryos were similar to those of oocytes, showing little change in permeability. In 8-cell embryos, the volume recovered much faster than in the earlier stages especially in glycerol and acetamide. In morulae, the volume recovery was much faster in glycerol and in ethylene glycol; in ethylene glycol, the extent of shrinkage was small and the recovery was fast, indicating an extremely rapid permeation. Although the permeability of oocytes/embryos generally increased as embryo development proceeded, the degree of increase varied greatly among the cryoprotectants. Interestingly, the volume change in propylene glycol was virtually unaffected by the stage of development. Such information will be valuable for determining a suitable protocol for the cryopreservation of oocytes/embryos at different stages of development.

Key words: Mouse, Embryo, Oocyte, Permeability, Cryoprotectant

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The survival of mammalian oocytes and embryos after cryopreservation varies with the stage of maturation and development. In general, oocytes/embryos at earlier stages appear to be more sensitive to cryopreservation. In the mouse, the rate of survival after cryopreservation is lower for unfertilized oocytes than embryos and increases as development proceeds up to the 8-cell or morula stage, although it may decrease as the blastocoel enlarges [1–3].

Several cryoprotectants have been shown to be effective for the preservation of mammalian oocytes and embryos, including dimethyl sulfoxide (DMSO) [1], glycerol [4], ethylene glycol [5], propylene glycol [6] and acetamide [7]. Cryoprotectants for mammalian oocytes and embryos are virtually limited to these five agents both in slow freezing and in vitrification [8]. Although the protective action of cryoprotectants is considered colligative [9], each agent has its own specific properties. Among them, the permeating property is of great importance, because permeation of the cell with a cryoprotectant is critical for the successful cryopreservation of

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mammalian oocytes/embryos. To prevent intracellular ice from forming, which is lethal to cryopreserved cells, permeation by a cryoprotectant and condensation are considered essential both in slow freezing and in vitrification. If the permeation is too slow, cells have to be exposed to the cryoprotectant either for a long time, at a high concentration, or at a higher temperature, which might expose the cells to the chemical toxicity of the cryoprotectant. Furthermore, cells are likely to be injured by osmotic over-swelling during the removal of the cryoprotectant after warming, if diffusion of the permeated cryoprotectant out of the cell is too slow. Generally, therefore, more permeating agents would be preferable.

When an oocyte or embryo is placed in a hypertonic solution containing 1–2 M cryoprotectant, it initially shrinks rapidly in response to the high extracellular osmolality, because diffusion of intracellular water out of the oocyte/embryo is faster than permeation of the cryoprotectant into the cell. After shrinking, the oocyte/embryo starts to regain its volume slowly as the cryoprotectant permeates the cell with water at a fixed osmolality. Consequently, it is possible to estimate the permeability of the oocyte/embryo to a cryoprotectant by measuring its volume changes. Leibo *et al.* [10] first adopted the volumetric approach to assess the permeability of embryos; they suggested that the permeation by DMSO of 8-cell mouse embryos is quite slow at 0 C. Mazur *et al.* [11] and Jackowski *et al.* [12] analyzed the permeability of mouse oocytes and embryos to glycerol quantitatively using a volumetric method. In a review paper, Mazur [13] stated that the permeability increased after fertilization, and further increased as the development proceeded from the 1-cell to 8-cell stage. Thereafter, several attempts were made to evaluate the rate at which cryoprotectants permeate oocytes or embryos at various stages in several species, though most of the studies investigated the permeability of oocytes/embryos at only one or two stages of maturation/development and for only one or two cryoprotectants.

In the mouse, several studies have examined the permeability of matured oocytes [14–20], whereas only a few studies have examined the permeability of embryos at the 1-cell [21], 8-cell [22], and 1-cell and morula [23] stages. One study compared the permeability of 1, 2, 4, and 8-cell embryos, but only for one cryoprotectant [24]. In bovine, permeability has been examined in oocytes [18, 25] or embryos at the morula and/or blastocyst stage [26–29]. In some species (e.g., human [14, 30–33], monkey [34], and goat [35, 36]), only oocytes have been assessed for permeability to cryoprotectants. Oocytes/embryos of other species in which the permeability has been assessed include rabbit morulae [37], equine blastocysts [38], sheep morulae [39], rat oocytes [40] and rat 1-cell embryos [41]. Therefore, the literatures contain no systematic comparison of the permeating properties of various cryoprotectants in relation to the developmental stages of oocytes/embryos.

In the present study, the permeating properties of five major cryoprotectants were examined using mouse oocytes at metaphase II and embryos at five developmental stages ranging from the 1-cell to the expanded blastocyst stage at room temperature. The change in volume of the oocytes and embryos was measured by precision method using a micromanipulator system, because their changes in volume are rapid.

Materials and Methods

Oocytes and embryos

Mouse oocytes at the metaphase II stage and embryos at the 1-cell, 2-cell, 8-cell, morula, and expanded blastocyst stages were used in the study. Mature female mice of the ICR strain (CLEA Japan Inc., Tokyo) were induced to superovulate with an intraperitoneal (i.p.) injection of 5 I.U. equine chorionic gonadotrophin (Serotropin, Teikokuzoki, Tokyo) followed by an i.p. injection of human chorionic gonadotrophin (hCG) (Puberogen, Sankyozoki, Tokyo) given 48 h later. For the collection of embryos, females were mated with mature males of the same strain. The females were sacrificed by cervical dislocation, and oviducts or uteri were removed. For the collection of oocytes, matured oocytes surrounded by cumulus cells were removed from the ampullar portion of the oviducts at 14 h after the injection of hCG from unmated animals. The oocytes were freed from cumulus cells by suspending them in PB1 medium [42] containing 0.5 mg/ml hyaluronidase (H-3506, Sigma, St Louis, MO, USA) followed by washing in fresh PB1 medium in a culture dish (Ø 35 × 10 mm)

under paraffin oil. For the collection of 1-cell, 2-cell and 8-cell embryos, oviducts of mated animals were flushed with PB1 medium at 25, 44, and 68 h, respectively, after the injection of hCG. For the collection of morulae, the uteri of mated animals were flushed with PB1 medium at 78 h after the injection of hCG. Some of the morulae were cultured in a modified Krebs-Ringer bicarbonate medium [43] under paraffin oil in a culture dish in a CO_2 incubator, and expanded blastocysts were collected after 18–24 h of culture.

Only oocytes and embryos of normal morphology were used in the experiments. Oocytes had a polar body, 1-cell embryos had polar bodies and pronuclei, and 2-cell embryos had two equal sized blastomeres. Eight-cell embryos were carefully examined for the presence of 8 distinct blastomeres, while morulae were examined for compaction. Expanded blastocysts had an apparent expanded blastocoel and zona pellucida, but were not hatching from the zona pllucida. The oocytes and embryos were washed and pooled in fresh PB1 medium in a culture dish under paraffin oil until each suite of experiments.

Measurement of volume change

The cryoprotectant solutions used were 10% v/v ethylene glycol, 10% v/v glycerol, 10% v/v DMSO, 10% v/v propylene glycol, and 1.5 M acetamide, all dissolved in PB1 medium. The osmolalities of the solutions were measured with osmometers based on the depression of the freezing point (OM 801, Vogel, Germany) and on the vapor pressure (Vapro 5520, Wescor Inc., Logan UT, USA), and are shown in Table 1.

In the cover of a Petri dish (Ø 94 × 10 mm), 200-μl drops of isotonic PB1 medium and cryoprotectant solutions were prepared under paraffin oil and placed on an inverted microscope. The temperature of the solution was monitored and the temperature of the room was adjusted so that the solution was kept at 25 C (± 0.5). In a micromanipulator system fixed to the microscope, a holding pipette (70–100 μm) and a covering pipette (150–200 μm) were set on the left and right arms, respectively (Fig. 1). The inner diameter of the holding pipette to which the oocyte/embryo was attached was small enough to prevent distortion of the oocyte/embryo. The covering pipette covered the oocyte/embryo together with the holding pipette. An oocyte or embryo was placed in PB1 medium and was held by the holding pipette. In the case of 2-cell embryos and 8-cell embryos, the embryo was oriented so that the blastomeres were clearly visible (Fig. 1A). Then, by sliding the dish, the pipette assembly holding the oocyte/embryo was introduced into a drop of cryoprotectant solution. By removing the covering pipette, the oocyte/embryo was exposed to the cryoprotectant solution instantly (Fig. 1B) [44]. A microscopical image of the oocyte/embryo, before and during the exposure to the cryoprotectant solution, was recorded by a time-lapse video tape recorder (ETV-820, Sony, Tokyo) every 0.5 sec for 25 min. From the images, the cross-sectional area of the oocyte/embryo in isotonic PB1 medium (0 sec) and at 5, 10, 20, 30, and 45 sec, and 1, 2, 3, 4, 5, 6, 8, 10, 15, 20 and 25 min after exposure to the cryoprotectants was measured. Then, the area relative to that in isotonic PB1 medium was calculated, and converted into the relative volume, on the assumption that the volume changes proportionally, using the equation $V=S^{3/2}$ where S is relative cross-sectional area and V is the relative volume. For each treatment, 3–4 oocytes/embryos were examined. The average relative volume was

Table 1. Osmolalities of PB1 medium containing a different cryoprotectant measured with two different osmometers

Cryoprotectant solution	Osmol/kg	
	Osmometer A*	Osmometer B*
1.5 M acetamide/PB1	1.98	1.70
10% v/v DMSO/PB1	2.09	1.73
10% v/v ethylene glycol/PB1	2.55	1.92
10% v/v glycerol/PB1	1.98	1.74
10% v/v propylene glycol/PB1	2.33	1.60
PB1 medium	0.291	0.294

* A: freezing point depression, B: vapor pressure.

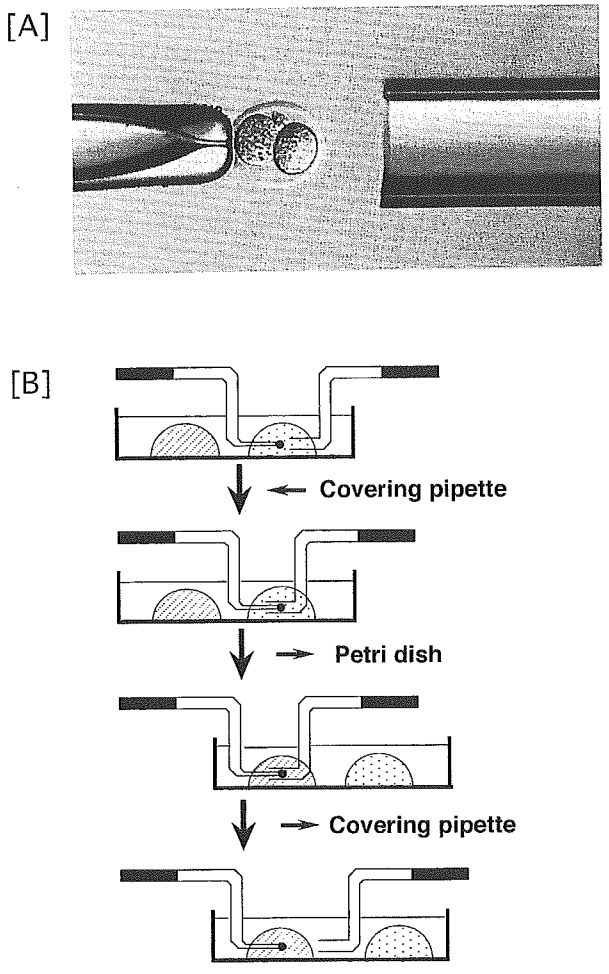

Fig. 1. The device and the procedure for direct transfer of an oocyte/embryo to a cryoprotectant solution using a micromanipulator system. [A] A photograph of a 2-cell embryo held by a holding pipette separate from the covering pipette on the right. [B] Schematic presentation of the device and the procedure with an oocyte/embryo (solid circle), a cryoprotectant solution (shaded hemisphere), and isotonic PB1 medium (dotted hemisphere).

plotted as a function of exposure time, to illustrate the permeating properties of each of the five cryoprotectants at each stage of development.

Results

Upon their transfer to the cryoprotectant solutions, the oocytes/embryos began to shrink quickly, reaching a minimal volume within 1 min of exposure, at all stages of development and in all the solutions. Then, they began to recover their volume slowly. However, the relative minimal volume and the recovery rate differed markedly between the stages of development and the cryoprotectants.

The average volume changes for oocytes during 25 min and the initial 5 min of exposure to the five cryoprotectants at 25 C are shown in Fig. 2. The least shrinkage and the fastest recovery of oocyte volume were observed in the propylene glycol solution, suggesting that propylene glycol permeated the oocytes the most rapidly. Oocytes in DMSO, acetamide, and ethylene glycol recovered much the same as the oocytes in propylene glycol, but slightly more slowly. On the other hand, oocytes exposed to glycerol had the largest extent of shrinkage and regained their volume extremely slowly, indicating that the permeability of oocytes to glycerol is quite low.

In two of the oocytes exposed to the acetamide solution, blebs formed and the oocytes were damaged after 6–8 min of exposure, therefore the graph in Fig. 2A ends at 6 min of exposure. This suggests that acetamide is toxic to mouse oocytes if used for a long period at 25 C.

The characteristic volume change of oocytes/embryos was manifested during a short period after suspension in the cryoprotectants, thus the data on embryos from the 1-cell to morula stage cover only 5 min of exposure. As shown in Fig. 3, the change in volume of 1-cell embryos was quite similar to that of oocytes, indicating that the permeability does not change at fertilization. The pattern of change essentially remained the same in 2-cell embryos. In 8-cell embryos, however, the volume recovered faster, especially in acetamide, ethylene glycol and glycerol. Notably, embryos suspended in the glycerol solution became more permeable, their recovery almost matching those of embryos exposed to the other cryoprotectants. In morulae, the recovery was much faster than in the earlier stages in ethylene glycol and glycerol; in ethylene glycol, the shrinkage was only slight and the embryos recovered their initial volume within 2 min of exposure (Fig. 3). This shows that ethylene glycol permeates morulae extremely rapidly.

Figure 4 shows the volume changes of expanded blastocysts during 25 min and the initial 5 min of exposure to five cryoprotectants at 25 C. The volume changes of expanded blastocysts could not be measured as accurately as those of other

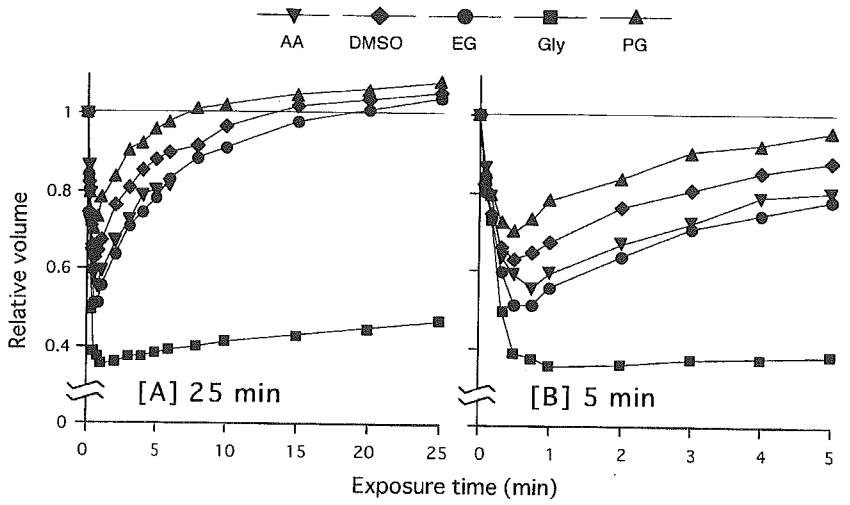

Fig. 2. The change in volume of mouse oocytes at the metaphase II stage during exposure to 1.5 M acetamide (AA), 10% DMSO, 10% ethylene glycol (EG), 10% glycerol (Gly), and 10% propylene glycol (PG), dissolved in PB1 medium, for [A] 25 min and [B] 5 min at 25 C.

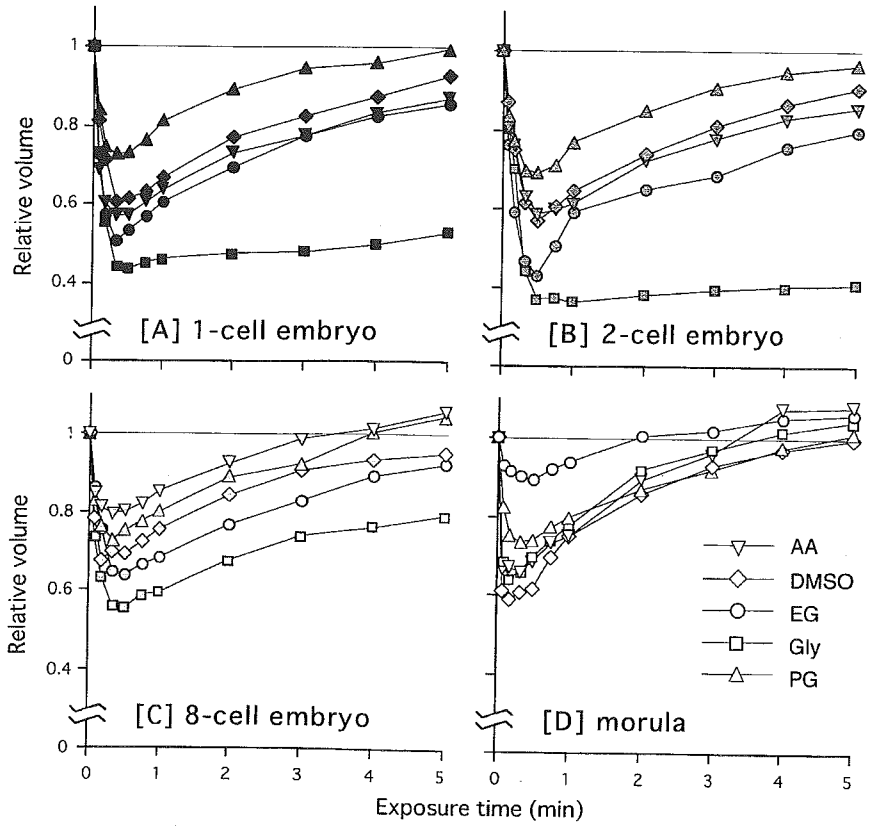

Fig. 3. The change in volume of mouse embryos at the [A] 1-cell, [B] 2-cell, [C] 8-cell and [D] morula stages during exposure to 1.5 M acetamide (AA, reversed triangles), 10% DMSO (diamonds), 10% ethylene glycol (EG, circles), 10% glycerol (Gly, squares), and 10% propylene glycol (PG, triangles), dissolved in PB1 medium, for 5 min at 25 C.

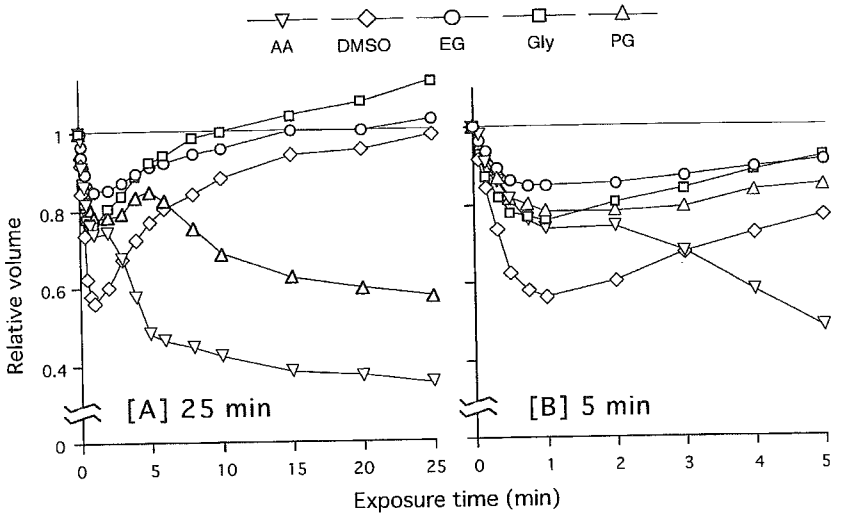

Fig. 4. The change in volume of expanded mouse blastocysts during exposure to 1.5 M acetamide (AA), 10% DMSO, 10% ethylene glycol (EG), 10% glycerol (Gly), and 10% propylene glycol (PG), dissolved in PB1 medium, for [A] 25 min and [B] 5 min at 25 C.

Table 2. Standard deviations (SD) of the relative volume for each type of oocyte/embryo after exposure to cryoprotectants for 1, 3 and 5 min

Time	Stage	Cryoprotectant*				
		AA	DMSO	EG	Gly	PG
1 min	Oocyte	0.062	0.017	0.006	0.069	0.066
	1 1-cell	0.079	0.042	0.044	0.026	0.035
	2-cell	0.027	0.028	0.140	0.038	0.044
	8-cell	0.014	0.049	0.041	0.063	0.009
	Morula	0.066	0.043	0.030	0.080	0.018
	Blastocyst	0.117	0.108	0.007	0.164	0.045
3 min	Oocyte	0.055	0.035	0.013	0.076	0.031
	1-cell	0.073	0.047	0.038	0.017	0.019
	2-cell	0.014	0.027	0.035	0.029	0.018
	8-cell	0.020	0.045	0.009	0.074	0.091
	Morula	0.077	0.044	0.052	0.048	0.032
	Blastocyst	0.144	0.063	0.008	0.149	0.060
5 min	Oocyte	0.065	0.016	0.033	0.069	0.030
	1-cell	0.067	0.029	0.043	0.012	0.020
	2-cell	0.037	0.019	0.027	0.056	0.011
	8-cell	0.025	0.042	0.003	0.086	0.030
	Morula	0.013	0.030	0.041	0.046	0.040
	Blastocyst	0.116	0.073	0.008	0.162	0.078

* AA: Acetamide, EG: ethylene glycol, DMSO: dimethyl sulfoxide, Gly: glycerol, and PG: propylene glycol.

oocytes/embryos, because the blastocysts had a large blastocoel and shrank unproportionally. Furthermore, the degree of expansion before measurement was not exactly the same among blastocysts. Therefore, standard deviation values for blastocysts, especially in acetamide and glycerol, were large (Table 2). Nevertheless, the results suggest that the permeability of blastocysts to ethylene glycol is similar to that of morulae, and the permeability to glycerol increased.

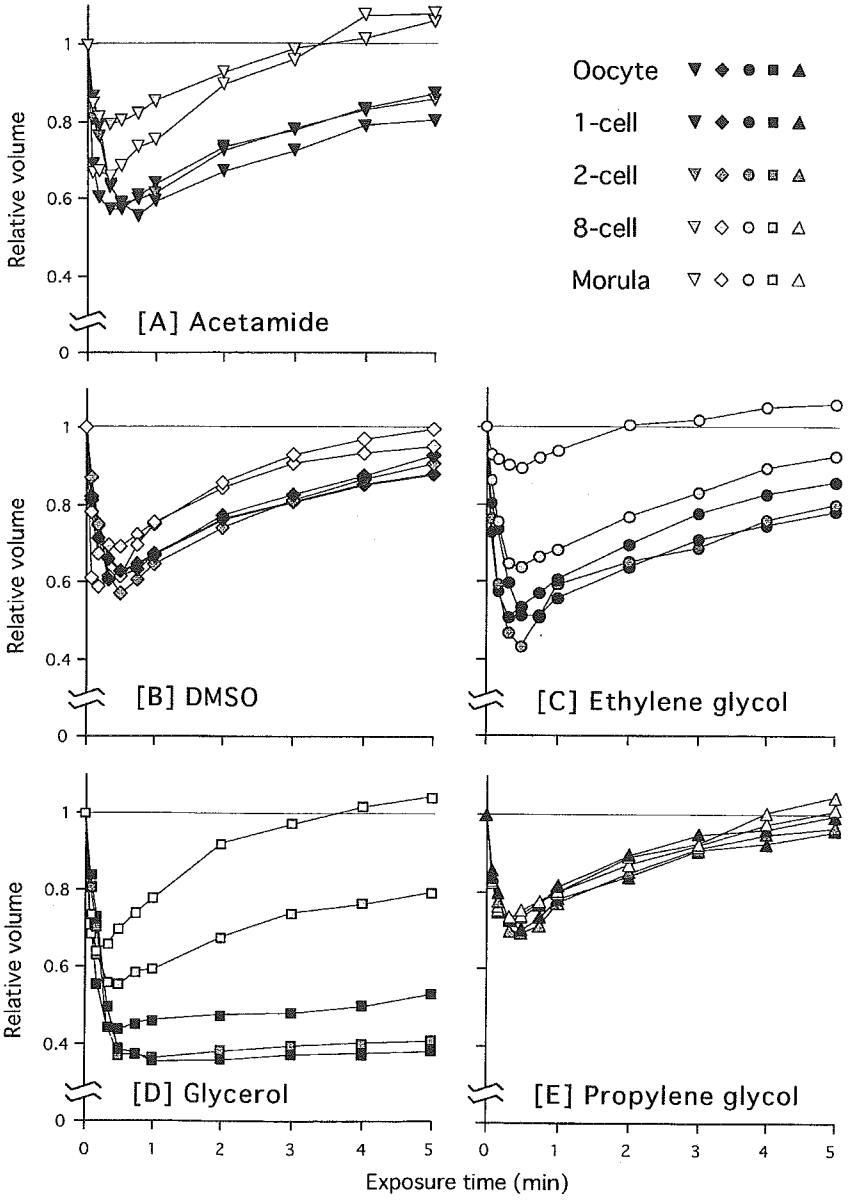

Fig. 5. The change in volume of mouse oocytes (at metaphase II), and embryos at the 1-cell, 2-cell, 8-cell and morula stages during exposure to [A] 1.5 M acetamide, [B] 10% DMSO, [C] 10% ethylene glycol, [D] 10% glycerol, and [E] 10% propylene glycol, dissolved in PB1 medium, for 5 min at 25 C. The figure was reconstructed from the data shown in Figs. 2 and 3. The darkness of the symbols represents the stage of the oocyte/embryo; the darkest symbols are for oocytes, and the darkness decreases as the development of embryo proceeds, with white symbols representing morulae.

Exceptionally, the recovery of volume ceased, and shrinkage occurred after 2–4 min of exposure in acetamide. In the propylene glycol solution, three of four blastocysts also stopped expanding and started to shrink at 5–8 min.

The average change in volume of oocytes and embryos, except expanded blastocysts, during 5 min of exposure was reconstructed for each cryoprotectant, and is shown in Fig. 5. It is clear that 1) the permeability of oocytes/embryos to

acetamide increases at the 8-cell stage; 2) the permeability of oocytes/embryos to ethylene glycol increases gradually until the 8-cell stage, and markedly at the morula stage; 3) glycerol is much less able to permeate oocytes up to the 2-cell stage, but becomes quite effective at the 8-cell stage and the morula stage; and 4) interestingly, the pattern of volume change of oocytes/embryos in propylene glycol was quite similar at all stages, showing that the permeability of oocytes and embryos to propylene glycol does not change during development.

Discussion

When an oocyte or an embryo is suspended in a drop of cryoprotectant solution from an isotonic solution, it first floats to the surface, and then sinks to the bottom. It is therefore difficult to record a focused microscopical image until it sinks after 1 or 2 min [10, 25, 27, 28]. However, the oocyte/embryo undergoes considerable volume excursion during the first few minutes of exposure, because the diffusion of water out of the cell upon exposure is very rapid. McGrath [45] devised a microscope diffusion chamber for direct real-time observation of the dynamic osmotic response of an individual cell on a dialysis membrane, in which the solution around the cell can be replaced by a perfused solution through the membrane. However, when evaluating the rate at which a solute permeates the cell, the rate at which the solute diffuses across the dialysis membrane must be taken into account. Gao *et al.* [46] devised a microperfusion method, in which an oocyte was held with a minute pipette in a minimal volume of isotonic solution under oil, and then a large volume of test solution was added. In this case, the oocyte would be exposed to a fast flow of test solution. In the present study, an oocyte/embryo was held by a holding pipette attached to a micromanipulator system and introduced into the cryoprotectant solution. Occasionally, when the oocyte/embryo was first introduced directly, it dropped off or was distorted during its passage through the oil. Therefore, the oocyte/embryo held by the holding pipette was covered with a covering pipette during its passage. Upon removal of the covering pipette, the small amount of isotonic PB1 medium around the oocyte/embryo would flow up instantly. Therefore, this method made it possible to expose an oocyte/embryo in an isotonic solution to a cryoprotectant solution instantly and smoothly in a fixed position for a microscope. This enabled accurate measurement of the volume change from the moment of exposure without any correction [44].

The results shown in Fig. 2 essentially reveal that oocytes are moderately permeable to propylene glycol, DMSO, acetamide and ethylene glycol, but less permeable to glycerol. This is consistent with reports on the permeability of mouse oocytes to propylene glycol [17], DMSO [15–17, 19, 24], ethylene glycol [19], and glycerol [12, 20].

However, it is not possible to compare the various results, because the conditions for volumetric assessment, e.g., the methods of measurement, the concentrations of the cryoprotectants, and the temperatures, vary among the reports. In some studies, the permeability coefficient for each cryoprotectant was calculated from a volume change curve. Therefore, it might be possible to compare the permeabilities in different reports quantitatively using the coefficient. However, several theoretical models have been devised to calculate the coefficient based on different assumptions [12, 15, 47], thus coefficients differ with the model even when the volume change is the same [24]. In the present study, therefore, we examined apparent volume changes of oocytes/embryos at six developmental stages in five cryoprotectants to enable direct comparison between stages and between cryoprotectants.

In comparison of the permeating properties of cryoprotectants based on the apparent volume change, the concentration of the cryoprotectants is an important factor. It would be preferable to use solutions with the same osmolality. Measurements of osmolality made with two types of osmometers using different measurement principles gave values which were roughly similar in all solutions. However, we decided they were not sufficiently similar (Table 1), so we used the same percentage by volume in the five cryoprotectant solutions (1.5 M acetamide approximately corresponded to 10% v/v). For more precise comparisons, a more accurate consideration of the cryoprotectant concentration will be necessary.

From the assessment of the volume change of mouse embryos at various developmental stages (Fig. 3), it was found that the permeability

remained virtually the same up to the 2-cell stage, but changed considerably at the 8-cell stage, especially for glycerol and acetamide, and at the morula stage for ethylene glycol and glycerol. Some reported data on the permeability of mouse embryos essentially agree with the present results; e.g., for 1-cell embryos exposed to ethylene glycol [21] and to DMSO [24], and for 8-cell embryos exposed to glycerol [13, 22]. Notably, Kasai [23] presented volume changes of 1-cell embryos and morulae for five cryoprotectants, which were quite similar to the present results, although the temperature was different (20 C). However, the results of a few reports are not consistent with the present results; e.g., Jackowski *et al.* [12] reported that the permeability of oocytes to glycerol increased after fertilization, and Pfaff *et al.* [24] reported that the permeability of embryos to DMSO decreased from the 4-cell stage to the 8-cell stage. No other comparative data are available.

Permeability generally increased as the development proceeded, although the degree of the increase and the transition stage differed between cryoprotectants (Fig. 5). At the 8-cell and morula stages, the permeation by ethylene glycol and glycerol increased dramatically, whereas the increase in permeability to DMSO was small. Furthermore, and interestingly, the permeating rate of propylene glycol was virtually unaffected by the stage of development. These characteristics definitely show that there exists a selective mechanism for the permeation by different agents.

The change in the pattern of permeation at the 8-cell and morula stages must be a reflection of certain physiological changes in the cell. Although the mechanism of change is not clear, the compaction of blastomeres might be involved because preparation for the following compaction would start at the 8-cell stage.

One of the major factors determining the permeating rate of a cryoprotectant is molecular weight (MW). Actually, the slower permeation by glycerol (MW=92.1) than ethylene glycol (MW= 62.1) of oocytes and embryos is reflected by the higher molecular weight, because these two agents belong to the same group of polyhydric alcohols. However, the similar or slightly higher permeability of mouse oocytes and 1-cell and 2-cell embryos to propylene glycol (MW=76.1) than to acetamide (MW=59.1) and ethylene glycol (MW= 62.1) is incompatible with the molecular weight being a determining factor in permeability. The molecular configuration might also affect the affinity for the membrane.

As a possible mechanism for the permeability, recent studies have suggested that water channels on the plasma membrane might be involved, because certain types of water channels, e.g., aquaporin 3 and aquaporin 7, can transport not only water but also glycerol, a representative cryoprotectant [44]. Actually, it has been shown that mRNAs of aquaporin 3 and aquaporin 7 are expressed in mouse oocytes and embryos [48], although the level of protein is not known. If such a channel actually affects permeation by glycerol, it might be reasonable to assume that the channel also transports ethylene glycol, a smaller polyhydric alcohol. However, it is not possible to account for the different patterns of permeation for other cryoprotectants with one type of water channel. Notably, the mechanism for the constant permeating pattern of propylene glycol throughout embryo development is difficult to explain. It might be that unknown channels which can transport propylene glycol selectively are expressed throughout the stages examined. Alternatively, propylene glycol might permeate the lipid bilayer rapidly.

Oocytes in acetamide formed blebs, and expanded blastocysts in acetamide and propylene glycol stopped expanding and began to shrink during exposure (Fig. 4). This unpredicted behavior of oocytes/embryos suggests that these cryoprotectants are more toxic than others. In toxicity tests for the same five cryoprotectants in mouse morulae, acetamide was the most toxic, followed by propylene glycol and then DMSO, whereas glycerol and ethylene glycol were much less toxic [49, 50].

There have been several reports on the permeability of mammalian oocytes and embryos to cryoprotectants. In matured oocytes, glycerol permeated more slowly than propylene glycol and DMSO in bovine [25], and glycerol permeated more slowly than ethylene glycol and propylene glycol in the goat [36], whereas the permeation by DMSO and ethylene glycol was similar in bovine oocytes [18]. In matured oocytes of humans and monkeys, DMSO [31, 32], propylene glycol [33], and ethylene glycol [34] permeated at moderate rates. In rat 1-cell embryos, propylene glycol permeated more rapidly than ethylene glycol and DMSO [41]. In

sheep morulae, ethylene glycol permeated more rapidly than propylene glycol, DMSO and glycerol [39]. In bovine blasotocysts, glycerol permeated more rapidly than DMSO [26]. All these observations are essentially consistent with the present results in mouse oocytes/embryos. Therefore, permeability may be affected more by the stage of development than the species, although a precise comparative study has not been conducted, except for our preliminary study on bovine oocytes and embryos [51]. We have examined the permeability of bovine oocytes and embryos to five cryoprotectants, as in the present study, and found that the pattern of permeation did not change from matured oocytes up to the 16-cell stage, but at the compaction stage (morula), the permeability to glycerol and ethylene glycol increased markedly. Although the pattern is not exactly the same as that of mouse oocytes/embryos, the results support the idea that permeability is affected more by the developmental stage in mammalian oocytes/embryos.

Although the mechanism of the change in the permeability to cryoprotectants is not known, the present study on five major cryoprotectants provides practical information for adopting a suitable cryoprotectant and for determining suitable conditions for impregnation and removal of the cryoprotectant at each stage of development in oocytes/embryos.

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Channel-Dependent Permeation of Water and Glycerol in Mouse Morulae[1]

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ABSTRACT

The cryosensitivity of mammalian embryos depends on the stage of development. Because permeability to water and cryoprotectants plays an important role in cryopreservation, it is plausible that the permeability is involved in the difference in the tolerance to cryopreservation among embryos at different developmental stages. In this study, we examined the permeability to water and glycerol of mouse oocytes and embryos, and tried to deduce the pathway for the movement of water and glycerol. The water permeability (LP, m min-1 atm-1) of oocytes and four-cell embryos at 25°C was low (0.63–0.70) and its Arrhenius activation energy (Ea, kcal/mol) was high (11.6–12.3), which implies that the water permeates through the plasma membrane by simple diffusion. On the other hand, the LP of morulae and blastocysts was quite high (3.6–4.5) and its Ea was quite low (5.1–6.3), which implies that the water moves through water channels. Aquaporin inhibitors, phloretin and p-(chloromercuri) benzene-sulfonate, reduced the LP of morulae significantly but not that of oocytes. By immunocytochemical analysis, aquaporin 3, which transports not only water but also glycerol, was detected in the morulae but not in the oocytes. Accordingly, the glycerol permeability (PGLY, × 10–3 cm/min) of oocytes was also low (0.01) and its Ea was remarkably high (41.6), whereas PGLY of morulae was quite high (4.63) and its Ea was low (10.0). Aquaporin inhibitors reduced the PGLY of morulae significantly. In conclusion, water and glycerol appear to move across the plasma membrane mainly by simple diffusion in oocytes but by facilitated diffusion through water channel(s) including aquaporin 3 in morulae.

embryo, developmental biology, ovum

INTRODUCTION

Since the first successful cryopreservation of mouse embryos in 1972 [1], various protocols have been developed to cryopreserve oocytes and embryos of many mammalian species. However, it is difficult to obtain high survival rates of embryos at different stages with a single cryopreservation protocol. For example, mouse morulae can be cryopreserved without appreciable loss of viability by a simple one-step vitrification method using an ethylene glycol-based solution [2], but pretreatment with a lower concentration of cryoprotectant is needed to vitrify mouse embryos at early stages [3]. This indicates that cryobiological properties differ among embryos at different developmental stages.

There are many factors that affect the survival of cells after cryopreservation [4]. Plasma membrane permeability is one factor determining the tolerance of cells to cryopreservation because the permeability modulates several major forms of cell injury caused by cryopreservation, i.e., injuries by intracellular ice formation, cryoprotectant toxicity, and osmotic swelling. Thus, it is likely that the plasma membrane permeability markedly differs among embryos at different developmental stages and with different cryosensitivities.

Water was long believed to move across the plasma membrane only by simple diffusion. In the 1990s, however, small intrinsic membrane proteins that act as water channels, called aquaporins (AQPs), were discovered and characterized [5]. We have already demonstrated that mRNAs of *Aqp3* and *Aqp7* are present in mouse oocytes at the metaphase II stage and embryos at the four-cell, morula, and blastocyst stages, and that mRNAs of *Aqp8* and *Aqp9* are expressed in embryos at the blastocyst stage [6]. Other researchers have also detected mRNAs of *Aqps* [7, 8] and AQP proteins [9] in mouse embryos. Thus, it is plausible that these AQPs play a role in the water permeability of mouse oocytes and embryos. However, it has not been shown whether such a channel pathway is a significant one in mouse oocytes and embryos.

The involvement of water channels in water movement across the plasma membrane can be deduced from the membrane's properties. In general, an osmotic water permeability higher than 4.5 μm min^{-1} atm^{-1} (or $P_f \geq 0.01$ cm/sec) and an Arrhenius activation energy (E_a) lower than 6 kcal/mol are suggestive of water movement principally through water channels, whereas an E_a higher than 10 kcal/mol is suggestive of movement principally via channel-independent diffusion [10]. Thus, it would be possible to deduce the pathway of movement from the water permeability and its E_a value.

If this criterion is applied to mature mouse oocytes, water channels may not be the major pathway for water movement across the plasma membrane because many studies have already shown that mouse oocytes have low water permeability (0.41–0.61 μm min^{-1} atm^{-1}) [11–16] and high E_a values (9.84–13.3 kcal/mol) [11–13, 17], although mouse oocytes have mRNAs of *Aqp3* and *Aqp7* [6]. They could be maternal stores of mRNA to be expressed later. Thus, most water molecules are assumed to move across the plasma membrane of oocytes by simple diffusion. In mouse embryos after cleavage, on the other hand, only a small number of studies have been available about the permeability of the plasma membrane. Pfaff et al. [16] estimated the water permeability of oocytes and embryos from the one-cell to eight-cell stage and demonstrated that eight-cell embryos are slightly more permeable to water than oocytes and one- to four-cell embryos, suggesting that water channels do not play a major role in

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water movement in embryos until the eight-cell stage. However, the water permeability of embryos at later stages has not been studied.

In this study, we first examined the water permeability of mouse oocytes and embryos and temperature dependence to deduce the involvement of water channels in water movement. Because the results of this experiment suggested the involvement of a water channel that can transport not only water but also glycerol in embryos at later developmental stages, we also examined the glycerol permeability of oocytes and morulae and temperature dependence to deduce the involvement of channels in glycerol movement.

MATERIALS AND METHODS

Collection of Oocytes and Embryos

Female ICR mice (CLEA Japan, Inc., Tokyo, Japan) were induced to superovulate with intraperitoneal injections of 5 IU of eCG and 5 IU of hCG given 48 h apart, and were mated with male ICR mice. Ovulated unfertilized oocytes were collected from the ampullar portion of the oviducts at 13 h after hCG injection without mating and were freed from cumulus cells by suspending them in modified phosphate-buffered saline (PB1) containing 37 U/ml hyaluronidase followed by washing with fresh PB1 medium. Four-cell embryos were flushed from the oviducts of mated animals with PB1 medium 55 h after hCG injection. Morulae were collected from the uteri by flushing them with PB1 medium 76 h after hCG injection, and only compacted morulae were used. To obtain early blastocysts, morulae collected 76 h after hCG injection were cultured in modified M16 medium supplemented with 10 μM EDTA, 1 mM glutamine, and 10 μM β-mercaptoethanol (modified M16 medium) [18], covered with paraffin oil for 6–8 h in a humidified CO_2 incubator equilibrated with 5% CO_2 in air at 37°C. Only early blastocysts, with a blastocoele larger than the inner cell mass and an unexpanded zona pellucida, were collected. To measure total cell volume of blastocysts, the blastocysts were pipetted repeatedly with a narrow-bore pipette to shrink their blastocoele. After being pipetted, early blastocysts with a shrunken blastocoele were incubated in modified M16 medium in a CO_2 incubator for 4–5 min and those without a re-expanded blastocoele were used for experiments. All experiments were approved by the Animal Ethics Committee of Kochi University.

Measurement of Water Permeability and Glycerol Permeability of Mouse Oocytes and Embryos

Water permeability was measured as described previously [19, 20]. Each oocyte or embryo was placed in a 100-μl drop of PB1 medium covered with paraffin oil in a Petri dish (90 × 10 mm) at 25°C and was held by a holding pipette (outer diameter being 80–120 μm) connected to a micromanipulator on an inverted microscope. The inner diameter of the holding pipette was small enough not to distort the oocyte or embryo. The temperature of the paraffin oil covering the various solutions was considered as the temperature of the solutions and was kept at 25 ± 1°C by controlling the temperature of the room. An oocyte or embryo held by a holding pipette was then covered with a covering pipette with a larger inner diameter (~200 μm) connected to another micromanipulator. Then, by sliding the dish, the oocyte or embryo was introduced into a drop of hypertonic PB1 medium containing sucrose (100 μl) at 25°C. By removing the covering pipette, the oocyte or embryo was abruptly exposed to the solution. The microscopic image of the oocytes and embryos during exposure to the solution was recorded with a time-lapse video tape recorder (ETV-820; Sony, Tokyo, Japan) every 0.5 sec. The cross-sectional area of oocytes and embryos was measured using an image analyzer (VM-50; Olympus, Tokyo, Japan). In the case of four-cell embryos, the cross-sectional area of only one blastomere in each embryo was measured. The cross-sectional area was expressed as a relative cross-sectional area, S, by dividing it by the area of the same oocyte and embryo in isotonic PB1 medium. The relative volume was obtained from $V = S^{3/2}$.

The osmotically inactive portion (V_b) of oocytes and embryos is required for the permeability analysis. To determine V_b, they were exposed to PB1 medium (0.295 Osm/kg) containing 0.110, 0.310, or 0.505 Osm/kg sucrose at 25°C for 60 min; the total osmolalities of the solutions were 0.405, 0.605, and 0.800 Osm/kg, respectively. Values for V_b were obtained from Boyle-van't Hoff plots.

The water permeability of oocytes and embryos was determined by measuring the shrinkage of oocytes and embryos after their transfer from isotonic PB1 medium to PB1 medium containing 0.505 Osm/kg sucrose (total osmolality, 0.800 Osm/kg) (sucrose/PB1) for 5 min at 25°C. In this study, we expressed water permeability as hydraulic conductivity (L_p). L_p values of the oocytes and embryos were determined by fitting water movement using a two-parameter formalism as described previously [19, 21]. The related constants and parameters used are listed in Table 1.

The L_p of oocytes and embryos was also measured at 15°C for 5 min, and the Arrhenius activation energy, E_a, or temperature dependence of L_p at each stage was obtained from Arrhenius plots.

In one experiment, oocytes and morulae were treated with phloretin or *p*-(chloromercuri) benzene-sulfonate (*p*-CMBS), which are AQP inhibitors, before water permeability was measured to study whether channels sensitive to these inhibitors were involved in water movement in oocytes and morulae. For this experiment, the water permeability of untreated oocytes and morulae was first measured at 25°C as described above, then the oocytes and morulae were transferred to PB1 medium and equilibrated for 10 min at 25°C. Next, they were treated with a water-channel inhibitor, 0.7 mM phloretin in PB1 medium for 2.5 min or 0.5 mM *p*-CMBS in PB1 medium for 30 min at 25°C. Finally, they were washed with PB1 medium, and water permeability was remeasured for 5 min at 25°C as described above.

The glycerol-permeability (P_{GLY}) of oocytes and embryos was determined by measuring the shrinkage and swelling of oocytes and embryos after transfer from isotonic PB1 medium to PB1 medium containing 10% (vol/vol) glycerol (1.565 Osm/kg) (total osmolality, 1.860 Osm/kg) (glycerol/PB1) for 20 min at 25°C. Values for S and V were obtained, and the P_{GLY} of the oocytes and embryos was determined by fitting water and solute movement using a two-parameter formalism as described above.

The P_{GLY} of oocytes and morulae was also measured at 15 and 25°C for 180 min, and the Arrhenius activation energy of the P_{GLY} for oocytes and morulae was obtained, as for the water permeability.

For the study of effects of AQP inhibitors on the glycerol permeability of morulae, the glycerol permeability of untreated morulae was first measured in glycerol/PB1 for 5 min at 25°C as described above, then the morulae were transferred to PB1 medium and equilibrated for 10 min at 25°C. Next, they were treated with a water-channel inhibitor in PB1 medium using the same conditions as for water permeability, washed with PB1 medium, and the permeability remeasured in glycerol/PB1 for 5 min at 25°C.

The osmolality of sucrose and glycerol (the osmolality of extracellular permeating solute) was calculated from published data about colligative properties of sucrose and glycerol in aqueous solutions [22]. The osmolality of PB1 medium (isotonic buffer) was measured with an osmometer (OM801; Vogel, Giessen, Germany).

In this report, we limited data collection to cells in the following volume ranges (before treatment): oocytes, 1.9–2.7 × 10E5 μm^3 (95% of all oocytes used); blastomeres of four-cell embryos, 0.5–0.7 × 10E5 μm^3 (92% of all four-cell embryos used); and morulae, 1.7–2.5 × 10E5 μm^3 (82% of all morulae used) because the equilibrated relative volume of extraordinarily larger or smaller oocytes and embryos in hypertonic sucrose solutions had smaller or larger V_b values than that of those with an ordinary size, suggesting that such extraordinarily large or small oocytes and embryos have different V_b values from ordinary ones (data not shown). For early blastocysts, however, we used all of the data because the size of blastocysts with a shrunken blastocoele varied greatly, probably due to the variable size of the blastocoele.

Expression of AQP3 and AQP7 in Oocytes and Morulae

For detection of AQP3, we used commercially available anti-human AQP3 goat antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), which cross-reacted with mouse AQP3. For AQP7, rabbit anti-mouse AQP7 serum was raised against a synthetic peptide (Asahi Techno Glass; Chiba, Japan) corresponding to the 15 C-terminal amino acids of mouse AQP7 (KNAASA-NISGSVPLE) (GenBank accession number AB010100). Using an ECL Western Blotting Kit (Amersham Biosciences Corp., Piscataway, NJ), we confirmed that the antiserum detected a 21-kDa protein in the membrane fraction of mouse kidney, which was consistent with the molecular weight of mouse AQP7, on a polyvinylidene fluoride membrane after electric transfer from an SDS-PAGE gel.

The zona pellucida of oocytes and morulae was removed by brief exposure to an acidic Tyrode solution (pH 2.5) [23]. The zona-free oocytes and morulae were fixed with a 2% paraformaldehyde solution containing 0.01 M sodium metaperiodate, 0.075 M lysine, and 0.075 M phosphate buffer (pH 7.4) at 4°C for 60 min. After being washed with PBS containing 5 mg/ml bovine serum albumin, they were permeabilized with PBS containing 0.25% Triton X-100. Then they were incubated with blocking solution; PBS containing 10% nonimmune donkey serum (Santa Cruz Biotechnology, Inc.) and 5 mg/ml bovine serum albumin (for AQP3) or 10% nonimmune goat serum (Santa Cruz Biotechnology, Inc.) and 5 mg/ml bovine serum albumin (for AQP7) at 25°C for 60 min. After being rinsed, they were incubated with diluted anti-human

AQP3 goat antibody (1/400) or anti-mouse AQP7 rabbit antiserum with blocking solution at 25°C for 60 min. As a control, instead of the primary antibodies, anti-human AQP3 goat antibody or anti-mouse AQP7 rabbit antiserum preincubated with a blocking peptide, which was the same peptide used for an antigen, at room temperature for 1 h, was used for the experiments. After being rinsed, the oocytes and embryos were incubated with diluted fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG donkey antibody (1/1600) (Santa Cruz Biotechnology, Inc.) (for AQP3) or diluted FITC-conjugated anti-rabbit IgG goat antibody (Chemicon International, Temecula, CA) (for AQP7) with blocking solution at 25°C for 30 min. They were observed under a fluorescence microscope. No staining was observed when primary antibodies preincubated with blocking peptides were used (data not shown). When cross sections of mouse kidney and testis that were fixed with paraformaldehyde, dehydrated with ethanol, embedded in paraffin, and cut at a thickness of 7 μm were stained with the same antibodies at the same dilution, the collecting duct or seminiferous epithelium was stained with anti-AQP 3 antibody or anti-AQP7 antibody, respectively, where the presence of AQP3 and AQP7 has been elucidated (data not shown) [24, 25].

RESULTS

Fraction of Osmotically Inactive Cell Content of Oocytes and Embryos

Figure 1 shows Boyle-van't Hoff plots of the relative volume of oocytes and embryos at various stages. The intercept of the regression line indicates osmotically inactive volumes for oocytes, four-cell embryos, morulae, and early blastocysts to be 15%, 16%, 15%, and 14%, respectively. We used these values to estimate the L_P and P_{GLY} of oocytes and embryos.

Water Permeability and Arrhenius Activation Energy of Oocytes and Embryos

In sucrose/PB1, oocytes and four-cell embryos shrank relatively slowly at 25°C, and shrank more slowly at 15°C than at 25°C (Fig. 2). On the other hand, morulae and early blastocysts shrank very rapidly at 15 and 25°C, the difference of the volume changes between the two temperatures being small (Fig. 2).

In oocytes and four-cell embryos, the value for L_P at 25°C was low (0.70 ± 0.12 and 0.63 ± 0.11 μm min^{-1} atm^{-1}) (Table 2) and the value for E_a of the L_P was high (12.3 and 11.6 kcal/mol) (Table 2), in which case water is expected to permeate through the plasma membrane predominately by simple diffusion [10]. In morulae, on the other hand, the average value for L_P at 25°C was quite high (4.45 ± 1.83 μm min^{-1} atm^{-1}), although the L_P value varied quite widely among embryos, from 2.04 to 9.35 μm min^{-1} atm^{-1} (data not shown). The value for E_a of the L_P was quite low (6.3 kcal/mol) (Table 2), in which case water is expected to move predominately through water channels [10]. In early blastocysts, L_P was also high (3.61 ± 1.72 μm min^{-1} atm^{-1}) and the value for E_a of the L_P was also quite low (5.1 kcal/mol) (Table 2). These results strongly suggest that most water molecules move across the plasma membrane by simple diffusion in oocytes and four-cell embryos but by facilitated diffusion through water channels in morulae and early blastocysts.

Effects of Water-Channel Inhibitors on Water Permeability of Oocytes and Morulae

To examine whether water permeates through water channels, we also examined the effect of water-channel inhibitors on the L_P of oocytes and morulae. As shown in Figure 3, neither phloretin nor *p*-CMBS affected the L_P of oocytes, whereas both inhibitors suppressed the L_P of morulae; treatment with phloretin and *p*-CMBS reduced the L_P value of morulae significantly from 4.24 ± 1.76 to 2.42 ± 0.94 μm

TABLE 1. Constant and parameters used for fitting permeability parameters.

Symbol	Meaning	Values
R	Gas constant	8.206×10^{-2} L atm $K^{-1}mol^{-1}$
T	Absolute temperatures	288 K and 298 K
$\overline{V_w}$	Partial molar volume of water	0.018 L/mol
$\overline{V_s}$	Partial molar volume of glycerol	0.071 L/mol[a]
V_b	Osmotically inactive fraction	—

[a] Value from Wolf et al. [22].

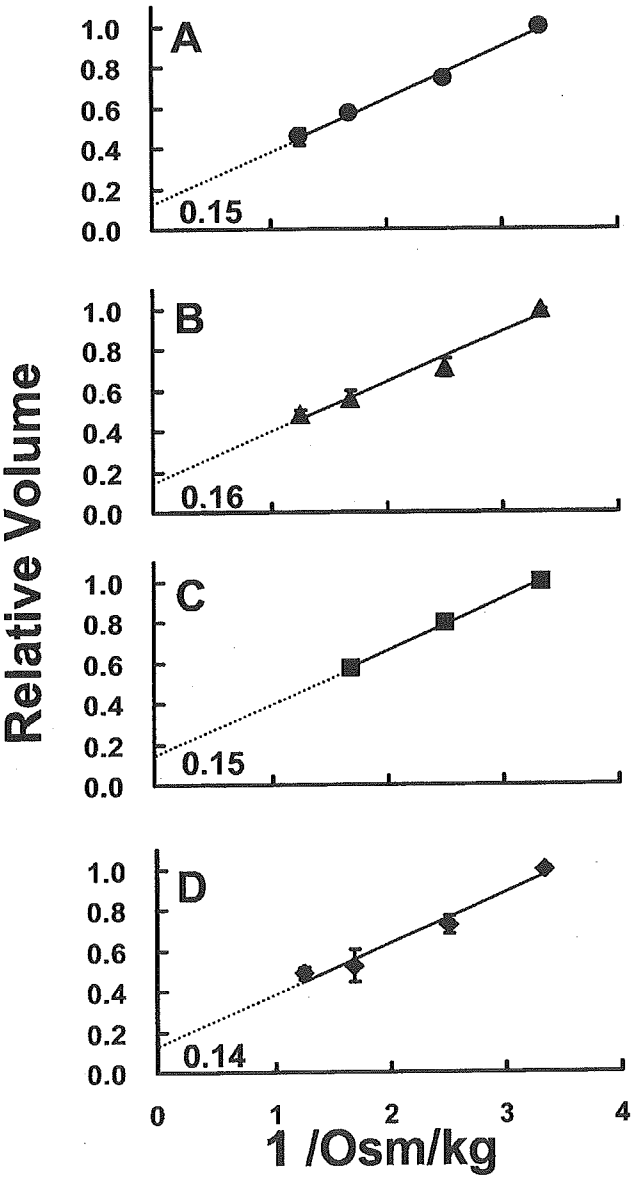

FIG. 1. Boyle-van't Hoff plots of mouse oocytes (**A**) and embryos at the four-cell (**B**), morula (**C**), and early blastocyst (**D**) stages. Oocytes (n = 5) and embryos at the four-cell (n = 5), morula (n = 4), and early blastocyst (n = 5) stages were exposed to various concentrations of sucrose in PB1 medium (0.405, 0.605, and 0.800 Osm/kg) at 25°C for 60 min, and relative volumes at 60 min were measured. Data are indicated as means of relative volume ± SD.

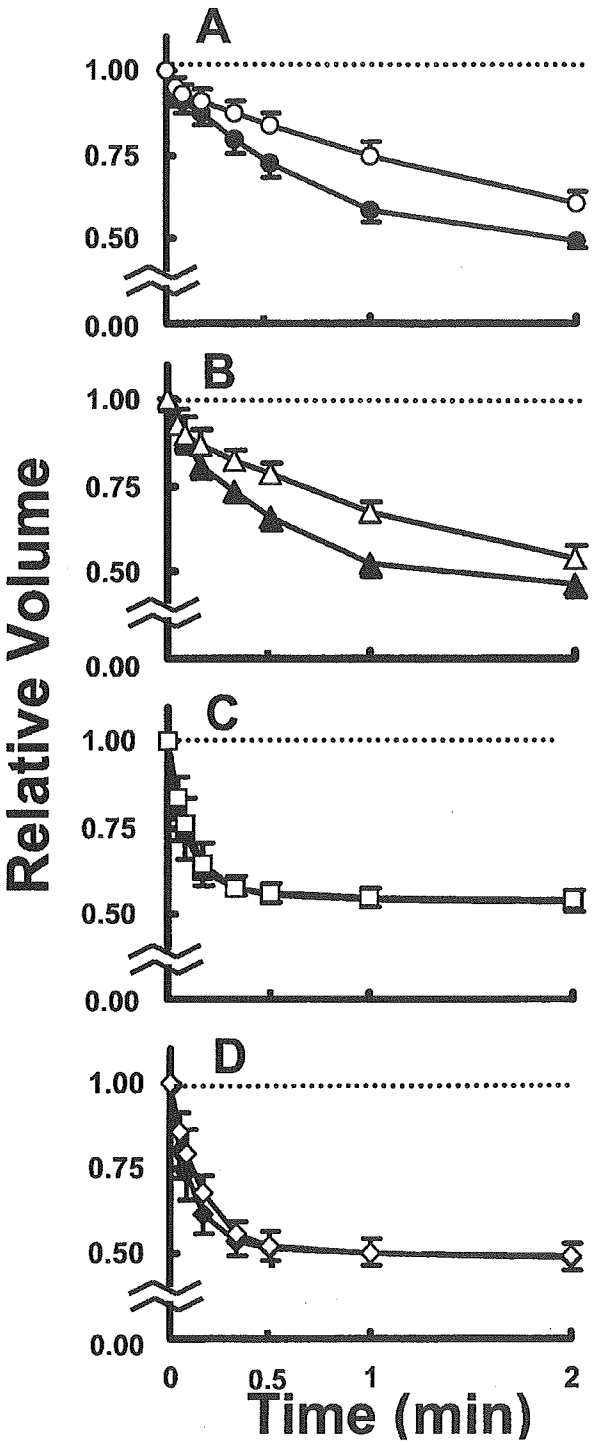

FIG. 2. Change in volume of mouse oocytes (**A**) and embryos at the four-cell (**B**), morula (**C**), and early blastocyst (**D**) stages in a hypertonic sucrose solution at 15°C (open symbols) and 25°C (closed symbols). Oocytes and embryos were exposed to sucrose in PB1 medium (0.800 Osm/kg) at 15 or 25°C for 5 min. Graphs show the volume changes during the first 2 min. Data are indicated as means of relative volume ± SD. Curves of oocytes and embryos at the four-cell, morula, and early blastocyst stages at 15°C are from 20 oocytes and 13, 26, and 22 embryos, respectively, and those at 25°C are from 27 oocytes and 17, 13, and 26 embryos, respectively.

TABLE 2. Hydraulic conductivity (L_p) and Arrhenius activation energy (E_a) of mouse oocytes and embryos.[a]

	L_p (μm $min^{-1}atm^{-1}$)[b]		
Oocytes/Embryos	15°C	25°C	E_a (kcal/mol)
Oocyte	0.34 ± 0.07	0.70 ± 0.12[c]	12.3
4-cell embryo	0.32 ± 0.08	0.63 ± 0.11[c]	11.6
Morula	3.07 ± 1.39	4.45 ± 1.83[d]	6.3
Blastocyst	2.68 ± 0.98	3.61 ± 1.72[d]	5.1

[a] Values are calculated from data in Figure 2.
[b] Values are expressed as means ± SD.
[c,d] Values with different superscripts within the same column are significantly different (Student *t*-test; $P < 0.01$).

min^{-1} atm^{-1} and from 3.31 ± 0.88 to 1.94 ± 0.75 μm min^{-1} atm^{-1}, respectively (Table 3). These results strongly suggest that phloretin- and *p*-CMBS-sensitive water channels are abundantly expressed in morulae and contribute to the permeation of water.

Immunofluorescence Staining of AQP3 and AQP7 in Oocytes and Morulae

Among mRNAs of the *Aqp* family, those of *Aqp3* and *Aqp7* have been detected in morulae in ICR mice [6]. So, it is plausible that AQP3 and/or AQP7 are abundantly expressed in morulae and that water permeates through these AQPs. Thus, we examined the expression of AQP3 and AQP7 in oocytes and morulae by an immunofluorescence technique (Fig. 4). The anti-AQP antibodies did not detect AQP3 and AQP7 in oocytes but detected the marked expression of AQP3 in morulae.

These results strongly suggest that rapid water movement in morulae relies on AQP3.

Glycerol Permeability of Oocytes and Embryos

Because AQP3 transports not only water but also glycerol [24], it is plausible that mouse morulae are highly permeable to glycerol. In glycerol/PB1 at 25°C, oocytes and four-cell embryos shrank relatively slowly and reached their minimal volume (35–42% of their isotonic volume) within 30 sec (Fig. 5), but regained a little of their volume after 20 min of exposure. On the other hand, morulae and early blastocysts shrank rapidly to 64% of their isotonic volume and then regained their volume after 3 min of exposure. The values for P_{GLY} of oocytes and four-cell embryos were low (0.01 ± 0.00 and 0.06 ± 0.01 × 10^{-3} cm/min, respectively), whereas the values of morulae and early blastocysts were remarkably high

TABLE 3. Hydraulic conductivity (L_p) of mouse oocytes and morulae treated with water-channel inhibitors.[a]

	L_p (μm $min^{-1}atm^{-1}$)[b]	
Inhibitors	Before treatment	After treatment
Oocyte		
Control	0.67 ± 0.14	0.63 ± 0.17
Phloretin	0.77 ± 0.15	0.86 ± 0.20
p-CMBS	0.70 ± 0.16	0.71 ±0.16
Morula		
Control	3.28 ± 1.32	3.06 ± 0.90
Phloretin	4.24 ± 1.76	2.42 ± 0.94*
p-CMBS	3.31 ± 0.88	1.94 ± 0.75*

[a] Values for control are calculated from 10 oocytes and 10 morulae, and other values are calculated from the data in Figure 3.
[b] Values are expressed as means ± SD.
* Significantly different from the L_p before treatment (Student *t*-test; $P < 0.01$).

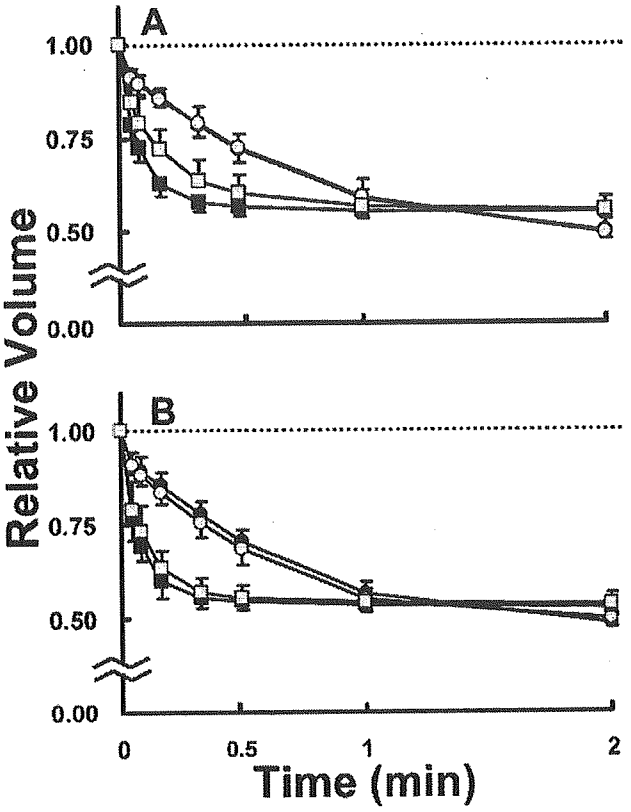

FIG. 3. Inhibition of water permeability of mouse oocytes and morulae by water-channel inhibitors. Oocytes (circles) and morulae (squares) were exposed to 0.7 mM phloretin in PB1 medium at 25°C for 2.5 min (**A**) (n = 10 and n = 10, respectively) or 0.5 mM *p*-(chloromercuri) benzenesulfonate in PB1 medium at 25°C for 30 min (**B**) (n = 12 and n = 11, respectively). Hydraulic conductivity was calculated from volume changes of oocytes and morulae in sucrose in PB1 medium (0.800 Osm/kg) at 25°C for 5 min before (closed symbols) and after (shaded symbols) treatment with the inhibitor. Graphs show the volume changes during the first 2 min. Data are indicated as means of relative volume ± SD. In (**A**), the untreated and treated oocyte data points fall on top of each other.

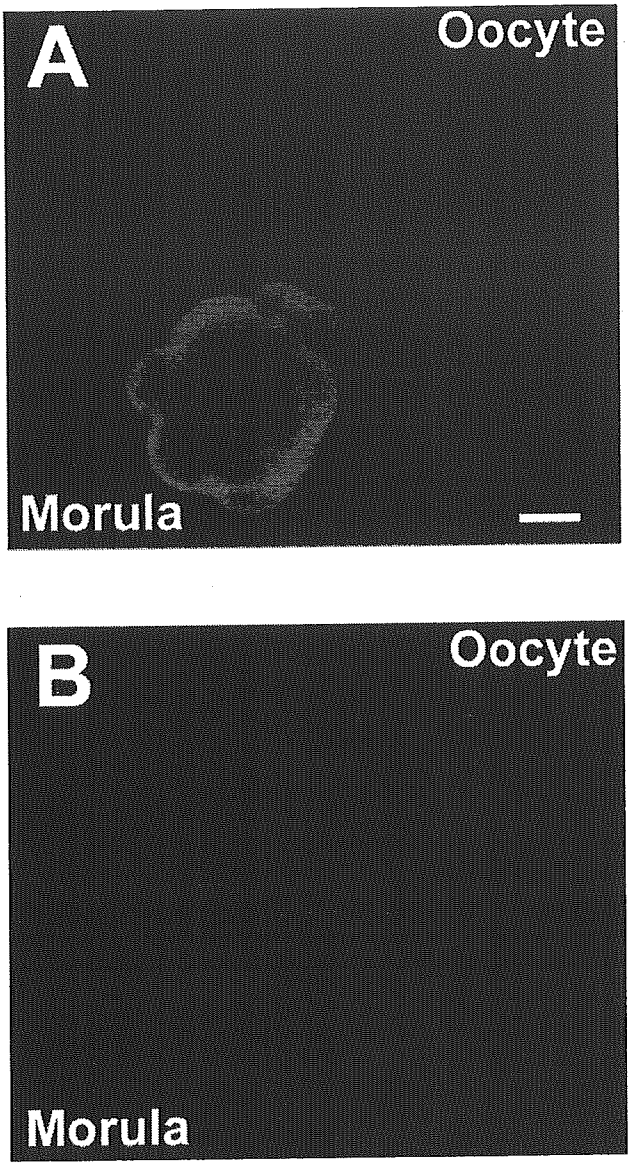

FIG. 4. Expression of aquaporin 3 (**A**) and aquaporin 7 (**B**) in a mouse oocyte and a morula. Expression of aquaporin proteins was detected by an immunofluorescence technique with anti-aquaporin 3 antibody (**A**) or anti-aquaporin 7 antiserum (**B**). Bar = 20 μm.

(4.63 ± 0.94 and 4.10 ± 0.73 × 10^{-3} cm/min, respectively), as in the case of L_p (Table 2).

These results support the hypothesis that AQP3 plays an important role in rapid water movement in morulae and suggest that AQP3 is also involved in rapid glycerol movement in morulae. The results also suggest that channels like AQP3 are involved in glycerol movement in blastocysts.

Arrhenius Activation Energy for Glycerol Permeability of Oocytes and Morulae

To elucidate the involvement of a channel process in glycerol movement in morulae, we examined the E_a for the P_{GLY} of oocytes and morulae. At 15°C, oocytes shrank and regained their volumes markedly more slowly than at 25°C, and they regained their volumes only slightly even after 180 min of exposure (Fig. 6A). On the other hand, morulae shrank and regained their original volumes only after 5 min of exposure at 15°C (Fig. 6B). The value of E_a for the P_{GLY} of oocytes was quite high (41.6 kcal/mol), whereas that of morulae was relatively low (10.0 kcal/mol) (Table 4), suggesting that glycerol permeates into oocytes mainly by simple diffusion whereas it permeates into morulae by facilitated diffusion through the plasma membrane.

Effects of Water-Channel Inhibitors on Glycerol Permeability of Morulae

To examine whether glycerol permeates through water channels in morulae, we examined the effect of water-channel inhibitors on P_{GLY} of morulae. As shown in Figure 7 and Table 5, both inhibitors suppressed the permeability of morulae; pretreatment with phloretin and *p*-CMBS reduced the P_{GLY} value of morulae significantly from 5.17 ± 1.56 to 3.15 ± 1.19 × 10^{-3} cm/min and from 5.11 ± 0.80 to 2.92 ± 1.13 ×

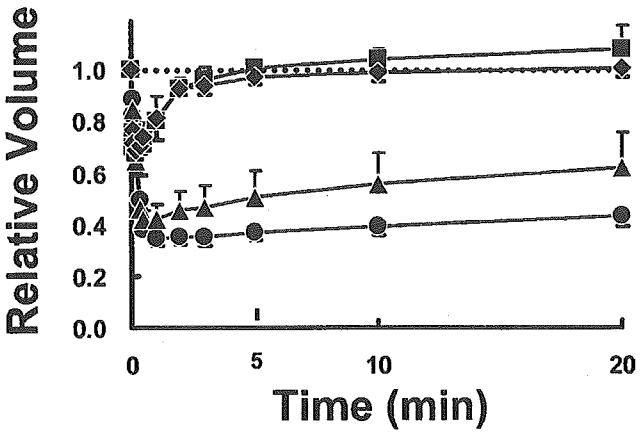

FIG. 5. Change in volume of mouse oocytes and embryos in 10% glycerol solution at 25°C. Mouse oocytes (circles) (n = 18) and embryos at the four-cell (triangles) (n = 7), morula (squares) (n = 14), and early blastocyst (diamonds) (n = 8) stages were exposed to 10% (vol/vol) glycerol in PB1 medium at 25°C for 20 min. Data are indicated as means of relative volume ± SD.

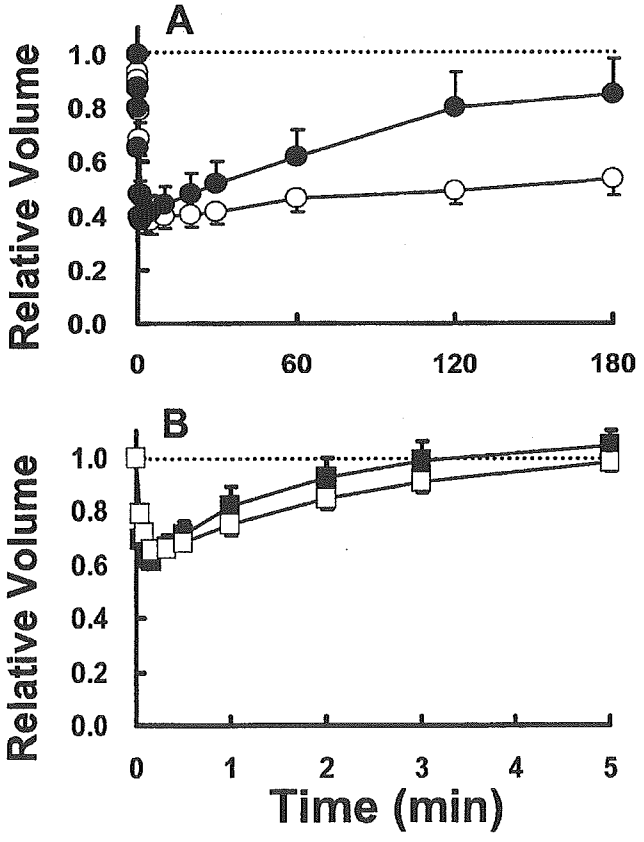

FIG. 6. Change in volume of mouse oocytes (**A**) and morulae (**B**) in 10% glycerol solution at 15°C (open symbols) and 25°C (closed symbols). Oocytes were exposed to 10% (vol/vol) glycerol in PB1 medium (1.89 Osm/kg) at 15 and 25°C for 180 min, and morulae were exposed to the solutions for 5 min. Data are indicated as means of relative volume ± SD. Each curve is from six oocytes or six morulae.

TABLE 4. Glycerol permeability (P_{GLY}) at 15 and 25°C and Arrhenius activation energy (E_a) of mouse oocytes and morulae.[a]

	P_{GLY} ($\times 10^{-3}$ cm/min)[b]		
Oocyte/Embryo	15°C	25°C	E_a (kcal/mol)
Oocyte	0.002 ± 0.001	0.023 ± 0.016[c]	41.6
Morula	2.610 ± 0.880	4.680 ± 1.500[d]	10.0

[a] Values are calculated from the data in Figure 6.
[b] Values are expressed as means ± SD.
[c,d] Values with different superscripts within the same column are significantly different (Student *t*-test; $P < 0.01$).

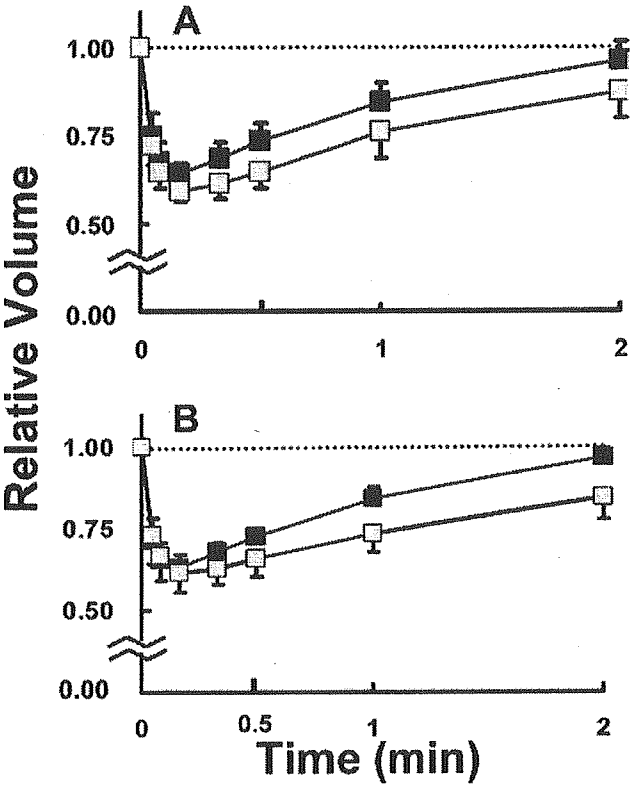

FIG. 7. Inhibition of glycerol permeability of mouse morulae by water-channel inhibitors. Morulae were exposed to 0.7 mM phloretin (**A**) or 0.5 mM *p*-(chloromercuri) benzensulfonate (**B**) in PB1 medium. Glycerol permeability was calculated from volume changes of morulae in 10% (vol/vol) glycerol in PB1 medium at 25°C for 5 min before (closed symbols) and after (shaded symbols) treatment with the inhibitor. Data are indicated as means of relative volume ± SD. Each curve is from 10 oocytes or 10 morulae.

TABLE 5. Glycerol permeability (P_{GLY}) of mouse morulae treated with water-channel inhibitors.[a]

	P_{GLY} ($\times 10^{-3}$ cm/min)[b]	
Inhibitors	Before treatment	After treatment
Control	4.62 ± 0.92	4.72 ± 0.83
Phloretin	5.17 ± 1.56	3.15 ± 1.19*
p-CMBS	5.11 ± 0.80	2.92 ± 1.13*

[a] Values for control are calculated from 10 morulae, and other values are calculated from the data in Figure 7.
[b] Values are expressed as means ± SD.
* Significantly different from the P_{GLY} before treatment (Student *t*-test; $P < 0.01$).

10^{-3} cm/min, respectively (Table 5). In control morulae, no difference was observed between the P_{GLY} values of first exposure and second exposure to glycerol/PB1.

These results suggest that phloretin- and *p*-CMBS-sensitive water channels and/or glycerol channels are abundantly expressed in mouse morulae and that the channels contribute to the permeation of the embryos by glycerol.

DISCUSSION

In this study, we show that the movement of water in mouse embryos at the morula stage relies on channel processes and suggest that the movement of glycerol in morulae also relies on channel processes.

Mature oocytes had low L_P and high E_a values (0.70 μm min^{-1} atm^{-1} and 12.3 kcal/mol, respectively) (Table 2). These values are similar to those of other studies [11–17] and suggestive of water movement by simple diffusion across the plasma membrane [10]. Four-cell embryos also had low L_P and high E_a values (0.63 μm min^{-1} atm^{-1} and 11.6 kcal/mol, respectively), similar to those of oocytes (Table 2). This L_P value is similar to that reported in another study (0.73 μm min^{-1} atm^{-1}) [16]. Thus, it appears that water also permeates through the plasma membrane of four-cell embryos primarily by simple diffusion.

On the other hand, morulae and early blastocysts had very high L_P and very low E_a values (4.45 and 3.61 μm min^{-1} atm^{-1} and 6.3 and 5.1 kcal/mol, respectively) (Table 2). The L_P value is close to 4.5 μm min^{-1} atm^{-1} and the E_a value is also close to 6 kcal/mol, suggesting the dependence of water movement on a channel pathway [10]. Thus, most water molecules would move across the plasma membrane through channel processes in morulae and blastocysts. Moreover, values for the L_P of morulae varied quite widely from 2.04 to 9.35 μm min^{-1} atm^{-1} among embryos (data not shown). The high variation in L_P can be explained by the different levels of channel expression among morulae but not by simple diffusion across the plasma membrane. Furthermore, AQP inhibitors did not affect the L_P of oocytes but significantly suppressed that of morulae (Table 3). All of these results strongly suggest that water channels are the major contributor to water movement in morulae and early blastocysts.

There has been no report that suggests that experimentally water-permeable channels other than AQPs expressed in the plasma membrane at physiological levels contribute to total plasma membrane water permeability [26]. Thus, AQPs may be the major contributor to the permeation of mouse morulae and early blastocysts by water.

We have already shown that mRNAs of *Aqp3* and *Aqp7* are present in mouse oocytes at the metaphase II stage and embryos from the four-cell to morula stages but those of other *Aqp*s were not in ICR mice [6]. Thus, these two AQPs may be involved in the marked increase in the L_P of the embryos at the morula stage. Offenberg et al. [7] and Offenberg and Thomsen [8] reported the expression of mRNAs of other *Aqp*s, including *Aqp1*, *Aqp5*, and *Aqp6*, in CD-1 X CB6F1/J embryos at various stages. The reason for this discrepancy is not known, but there may be mouse strain-specific differences in *Aqp* mRNA subtypes present.

It is known that mercuric compounds, such as $HgCl_2$ and *p*-CMBS, inhibit various AQPs, including AQP1, AQP2, and AQP3, by binding to a critical sulfhydryl [27], but not AQP7 because it does not have a critical sulfhydryl [25]. Phloretin is known as a urea-transporter inhibitor and inhibits the transport of water by AQP3 [24, 28], although its effect on AQP7 is not known. Thus, it was expected that phloretin and *p*-CMBS would decrease the L_P of morulae if AQP3 was dominantly expressed in morulae, whereas the inhibitors might not affect the L_P if AQP7 was dominantly expressed. Both phloretin and *p*-CMBS significantly reduced the L_P of morulae (Fig. 3 and Table 3), supporting the former assumption that AQP3 was expressed dominantly in morulae.

This hypothesis was confirmed by detection of the marked expression of AQP3 protein on the apical side of blastomeres of morulae but not in oocytes by an immunofluorescence technique (Fig. 4A). On the other hand, little expression of AQP7 was detected in oocytes or morulae (Fig. 4B). Thus, AQP3 must be the major contributor to water transport in morulae.

Barcroft et al. [9] also reported that AQP3 was detected markedly on the apical side of blastomeres of morulae of CD-1 X CB6F1/J mice. Moreover, they did not detect other AQPs on the apical side of blastomeres of the morulae, although they detected other AQPs in the region of cell-cell contact. Thus, in morulae, the major water pathway must be AQP3 in the mouse.

In early blastocysts, the additional expression of mRNAs of *Aqp8* and *Aqp9*, in addition to those of *Aqp3* and *Aqp7*, has also been observed in ICR mice [6]. Barcroft et al. [9] showed in CD-1 X CB6F1/J embryos that expression of AQP3 became restricted in the inner cell mass and the basolateral cell margins of the trophectoderm and that AQP9 was expressed in the inner cell mass and the apical membrane of the trophectoderm. If the AQPs are distributed in blastocysts of ICR mice similarly, AQPs other than AQP3 could play a role in water movement in the blastocysts. Because the blastocysts we used were pipetted repeatedly to shrink their blastocoele and thus may have had small rips in their trophectoderm from the pipetting, it is possible that water moved not only across the apical side but also across the blastocoele-side of the plasma membrane in hypertonic sucrose solution. Thus, AQPs expressed in the inner cell mass and the basolateral cell margins of the trophectoderm might partially contribute to the water movement in our experiments. Further studies are needed to clarify which type of AQP is involved in water movement in mouse blastocysts.

AQP3 is an aqua glyceroporin, which can transport not only water but also neutral solutes with a low molecular weight, such as glycerol [24]. Thus, we examined the P_{GLY} of mouse oocytes and embryos (Fig. 5). The P_{GLY} of oocytes and four-cell embryos was low (0.01–0.06 $\times$ 10^{-3} cm/min), whereas that of morulae was high (4.63 $\times$ 10^{-3} cm/min), being more than 100 times higher than that of oocytes. This result suggests that AQP3 plays a role in the marked increase in the permeability of morulae to glycerol.

The pioneering studies by Mazur and his group [29, 30] demonstrated that the permeability of mouse embryos to glycerol increased from oocytes and one-cell zygotes to eight-cell embryos. The P_{GLY} values of oocytes and four-cell embryos in their studies (0.01 and 0.05 $\times$ 10^{-3} cm/min, respectively, at 20–22°C) are almost the same as those in the present study (0.01 and 0.06 $\times$ 10^{-3} cm/min, respectively, at 25°C). They also reported that a marked increase in the P_{GLY} of embryos was observed at the eight-cell stage (0.34 $\times$ 10^{-3} cm/min). In the present study, we observed further remarkable increase in the P_{GLY} at the morula stage (4.63 $\times$ 10^{-3} cm/min at 25°C). Thus, the marked increase in the expression of glycerol-permeable channels appears to begin at around the stage when mouse embryos develop to compacted morulae.

Thus, we studied the E_a for P_{GLY} and the sensitivity of P_{GLY} to AQP inhibitors in oocytes and morulae. The value of E_a for the P_{GLY} of oocytes was quite high (41.6 kcal/mol), whereas that of morulae was much lower (10.0 kcal/mol) (Table 4). The marked decrease in E_a suggests that channel processes play a

role in rapid glycerol movement in morulae. Moreover, phloretin and *p*-CMBS suppressed the P_{GLY} of morulae significantly (Table 5), suggesting that channels sensitive to these inhibitors were involved in glycerol movement in morulae. It has been shown that *p*-CMBS also suppresses the P_{GLY} of AQP3 [24, 28]. On the other hand, phloretin suppresses the L_p [24, 28] but not P_{GLY} of AQP3 [28]. Thus, rapid glycerol movement in morulae should rely partly on AQP3, but other glycerol channels might be involved in the process.

Early blastocysts also had remarkably high glycerol permeability, like the morulae (Fig. 5), suggesting that glycerol movement in blastocysts also relies on channel processes. It has been reported that the permeation by glycerol into CD-1 X CB6F1/J blastocysts is suppressed by *p*-CMBS [9]. Thus, mouse blastocysts also have mercury-sensitive channel-dependent glycerol pathways, the same as morulae. Further studies are needed to clarify which channels are involved in glycerol transport in blastocysts.

We have already examined changes in volume of mouse oocytes and embryos in various cryoprotectant solutions in preliminary experiments and showed that the pattern of cryoprotectant permeation does not change from matured oocytes up to embryos at the two-cell stage, but permeability to glycerol and ethylene glycol drastically increases at around the morula stage [20]. In bovine oocytes and embryos, we have also observed similar changes; the pattern of cryoprotectant permeation does not change from matured oocytes up to embryos at the 16-cell stage, but permeability to ethylene glycol and glycerol drastically increases at the morula stage [31]. Thus, the marked increase in aqua glyceroporins, such as AQP3, in the later stages of development may occur not only in the mouse but also in other mammalian species. Such increased permeability of mammalian embryos might play a role in the uptake of small molecules from tubal or uterine fluid or in the excretion of metabolites from embryos. However, the role appears not to be vital for the development of mouse embryos because *Aqp3* knockout mice can develop to term and are grossly normal except for polyurea [32]. However, because aqua glyceroporins are permeable to various cryoprotectants, the expression may also affect the tolerance of embryos at different developmental stages to cryopreservation. Thus, the present study provides important information for understanding the cryobiological properties of mammalian oocytes and embryos and for formulating cryopreservation protocols for mammalian oocytes and embryos.

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Use of Frozen-Thawed Oocytes for Efficient Production of Normal Offspring from Cryopreserved Mouse Spermatozoa Showing Low Fertility

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Freezing of spermatozoa and unfertilized oocytes is a useful tool for the conservation of mouse genetic resources. However, the proportion of frozen-thawed oocytes fertilized with spermatozoa in vitro is low because spermatozoa, especially those frozen-thawed, can not penetrate into oocytes because of hardening of the zona pellucida following premature release of cortical granules. To produce offspring efficiently from cryopreserved transgenic mouse gametes, we fertilized frozen-thawed gametes by using intracytoplasmic sperm injection (ICSI) and assessed pre- and postimplantation development of embryos. Compared with fresh unfertilized oocytes, frozen-thawed unfertilized oocytes were highly tolerant to damage by injection, as the survival rates after injection of frozen spermatozoa were 51 and 78%, respectively. Frozen-thawed oocytes that survived after sperm injection developed normally to the blastocyst stage and gave rise to offspring. Moreover, offspring with transgenes also were obtained from frozen gametes fertilized by ICSI. These results demonstrate that ICSI is an efficient technique for producing offspring from transgenic spermatozoa showing low fertility and that use of frozen-thawed oocytes leads to conservation of genetic resources because suboptimally preserved gametes are not wasted.

Freezing of mouse sperm by using a solution containing raffinose and skim milk (25) has been widely used with various mouse strains (17). Moreover, an in vitro fertilization (IVF) method using frozen spermatozoa has been adopted for large-scale conservation of new mouse lines, such as mutant mice generated by treatment with N-ethyl-N-nitrosourea (13, 26). To produce embryos by IVF, researchers typically must prepare a large number of fresh unfertilized oocytes for each experiment. Providing they show normal development after fertilization, the use of frozen-thawed oocytes would save time and effort during preparation for IVF, and a minimal number of embryos sufficient for each experiment could be produced from frozen gametes. Furthermore, various combinations of embryos could be produced from preserved gametes if oocytes and spermatozoa of many mouse strains were frozen separately (15).

Although frozen-thawed oocytes could be fertilized with fresh (31) and frozen-thawed spermatozoa (15) in vitro, both fertilization rates were low. Carroll and colleagues (2) suggested that the low fertility rate of frozen-thawed oocytes is caused by the hardening of the zona pellucida due to the premature release of cortical granules after freezing. Today, advances in producing induced mutant mice have led to the production of a large number of new mouse lines from C57BL/6J inbred mice. However, the fertilization rate of C57BL/6J spermatozoa after freeze-thawing was poor, even when fresh oocytes were used (19, 22, 24). Although it was inefficient to fertilize frozen-thawed oocytes with frozen-thawed spermatozoa in vitro, recent reproductive technologies such as partial zona dissection (18) and intracytoplasmic sperm injection (ICSI) (10, 12) are powerful tools to increase the efficiency of fertilization. The advantage of ICSI is that only one spermatozoon is required to fertilize an oocyte, thereby preventing wasting of valuable preserved gametes. In this study, we used ICSI of frozen-thawed spermatozoa to fertilize frozen-thawed unfertilized oocytes and assessed the developmental potential of these embryos.

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Materials and Methods

Animals. All animals were obtained from CLEA Japan, Inc. (Tokyo, Japan). C57BL/6J Jcl male (age, > 10 weeks) and female (age, 8 to 12 weeks) mice were used for sperm and oocyte donors, respectively. Jcl:ICR female mice (age, 8 to 16 weeks) were the recipients of two-cell embryos. All animals were maintained in an air-conditioned (22°C) and light-controlled (12:12-h light:dark cycle; lights on, 7 a.m.) room. Microbial monitoring for mouse hepatitis virus, Sendai virus, *Citrobacter rodentium*, *Clostridium piliforme*, *Corynebacterium kutscheri*, *Helicobacter bilis*, *Helicobacter hepaticus*, *Mycoplasma* spp., *Pasteurella pneumotropica*, *Salmonella* spp., *Aspiculuris tetraptera*, *Syphacia* spp., *Giardia muris*, *Spironucleus muris*, trichomonads, and ectoparasites was done monthly; all animals had negative test results. The Animal Care and Use Committee of the Kumamoto University School of Medicine approved all procedures performed in this study.

Media. All chemicals were obtained from Nacalai Tesque, Inc. (Kyoto, Japan) unless otherwise stated. Human tubal fluid (HTF) medium (21) was used for oocytes collection before freezing. PB1 medium (30) was used as a solution to dissolve cryoprotectants